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FORM PTO-1390 (Modified) 2000		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 20363-012	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371					
INTERNATIONAL APPLICATION NO. PCT/US00/15325		INTERNATIONAL FILING DATE 02 June 2000 (02.06.2000)		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 10/009584 PRIORITY DATE CLAIMED 04 June 1999 (04.06.1999)	

TITLE OF INVENTION
IDENTIFICATION OF COMPOUNDS THAT MODIFY TRANSCRIPTIONAL RESPONSES TO HYPOXIA

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau)
 - b. ☐ have been communicated by the International Bureau
 - c. ☐ have not been made, however, the time limit for making such amendments has NOT expired
 - d. ☒ have not been made and will not be made
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4))
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4)
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Express Mail Label No. EL862267031US
Filed On: 04 December 2001 (04.12.01)

U.S. APPLICATION NO. 10/009584 37 CFR		INTERNATIONAL APPLICATION NO. PCT/US00/15325		ATTORNEY'S DOCKET NUMBER 20363-012	
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24. The following fees are submitted.. BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 <div style="text-align: right;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS PTO USE ONLY	
				\$690.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	24 - 20 =	4	x \$18.00	\$72.00	
Independent claims	5 - 3 =	2	x \$80.00	\$160.00	
Multiple Dependent Claims (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL OF ABOVE CALCULATIONS =				\$1,052.00	
<input type="checkbox"/> Applicant claims small entity status (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				\$0.00	
SUBTOTAL =				\$1,052.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$1,052.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)) The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <input type="checkbox"/>				\$0.00	
TOTAL FEES ENCLOSED =				\$1,052.00	
				Amount to be: refunded	\$
				charged	\$

a. ☒ A check in the amount of **\$1,052.00** to cover the above fees is enclosed

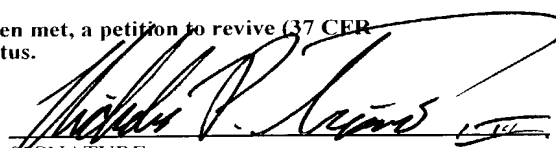
b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0311** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO.

ELRIFI, Ivor R. Mintz, Levin, Cohn, Ferris, Glovsky & Popeo, P.C. One Financial Center Boston, Massachusetts 02111 United States of America	<div style="text-align: center;">  SIGNATURE </div> <div style="text-align: center;"> TRIANO, Nicholas P. III NAME </div> <div style="text-align: center;"> 36,397 REGISTRATION NUMBER </div> <div style="text-align: center;"> 04 December 2001 (04.12.01) DATE </div>
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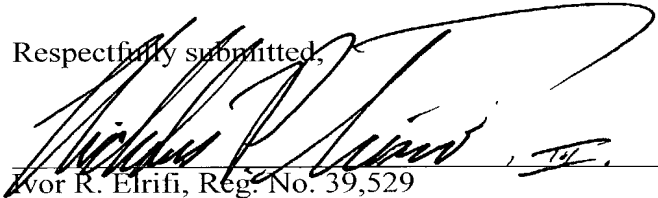
REMARKS

Applicants have amended the claims herein so as to clarify and more particularly indicate the claimed subject matter. The cancellation of and/or amendments to the claims are being made solely to expedite prosecution of this application. Applicant reserves the option to further prosecute the same or similar claims in this or in another patent application. No new matter is added. *After entry of this amendment, claims 1-24 are in the case.*

CONCLUSION

On the basis of the foregoing amendments, Applicants respectfully submit that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact either of the undersigned at the telephone number provided below.

Respectfully submitted,



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Dated: December 4, 2001

Appendix A: marked up version of the claims showing the changes made

10. (amended once) The method of claim 8[or 9], wherein said first transcription factor comprises a peptide of HIF-1 α and said second transcription factor comprises a peptide of p300/CBP.

TRA 1602436v1

IDENTIFICATION OF COMPOUNDS THAT MODIFY
TRANSCRIPTIONAL RESPONSES TO HYPOXIA

5 Statement as to Federally Funded Research

This work was supported, in part, by grants from the United States government (grants CA15751 and 5K08CA72993 from the National Institutes of Health). The government has certain rights in the invention.

Background of the Invention

10 This invention relates to methods for identifying compounds that modify transcriptional responses to hypoxia.

As solid tumors evolve from a single malignant cell into a multicellular mass, oxygen tension in the tumor microenvironment drops as the passive diffusional capacity of the existing blood supply is surpassed
15 (Nordsmark *et al.*, *Acta. Oncol.* 33:383-389, 1994; Helmlinger *et al.*, *Nat. Med.* 3:177-182, 1997; Adam *et al.*, *Head Neck* 21:146-153, 1999). Tumor hypoxia decreases the efficacy of many common therapies (Brown, *Mol. Med. Today* 6:157-162, 2000), is a powerful stimulus for angiogenesis (Shweiki *et al.*, *Nature* 359:843-845, 1992), and is linked to tumor progression (Zhong *et al.*,
20 *Cancer Res.* 59:5830-5835, 1999; Hockel *et al.*, *Cancer Res.* 56:4509-4515, 1996) and metastasis (Brizel *et al.*, *Cancer Res.* 56:941-943, 1996). Since chronic hypoxia differentiates tumor cells from their normal tissue counterparts (Zhong *et al.*, *Cancer Res.* 59:5830-5835, 1999), therapeutic strategies have been developed that seek to exploit this difference by the design of drugs that
25 are selectively activated under hypoxic conditions (Zeman *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.* 12:1239-1242, 1986) and hypoxia-induced gene therapy

protocols (Binley *et al.*, Gene Ther. 6:1721-1727, 1999; Shibata *et al.*, Gene Ther. 7:493-498, 2000).

Cellular hypoxia triggers a multifaceted adaptive response that is primarily mediated by the heterodimeric transcription factor, HIF-1 (MacDonald *et al.*, Mol. Cell. Biol. 13:5907-5917, 1993; Maltepe *et al.*, Nature 386:403-407, 1997). Under normoxic conditions, the alpha subunit (HIF-1 α) is essentially undetectable due to its destruction by the ubiquitin-proteasome system (Salceda *et al.*, J. Biol. Chem. 272:22642-22647, 1997; Huang *et al.*, J. Biol. Chem. 271:32253-32259, 1996). This is achieved, in part, through targeting by the von Hippel-Lindau protein (Huang *et al.*, J. Biol. Chem. 271:32253-32259, 1996). Under hypoxic conditions, through as yet unclear mechanisms, HIF-1 α is stabilized and accumulates (Jiang *et al.*, Am. J. Physiol. 271:C1172-1180, 1996). Stabilized HIF-1 α heterodimerizes with its binding partner, ARNT (aryl hydrocarbon receptor nuclear translocator) (Wang *et al.*, J. Biol. Chem. 270:1230-1237, 1995; Jiang *et al.*, J. Biol. Chem. 271:17771-17778, 1996), a common binding partner of several β HLH-PAS domain proteins. The heterodimer interacts with p300/CBP (Arany *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:12969-12973, 1996; Ebert *et al.*, Mol. Cell. Biol. 18:4089-4096, 1998; Kallio *et al.*, EMBO J. 17:6573-6586, 1998; Carrero *et al.*, Mol. Cell. Biol. 20:402-415, 2000) and SRC-1 family coactivators (Carrero *et al.*, Mol. Cell. Biol. 20:402-415, 2000), binds to a cognate hypoxia-response element (HRE) (Jiang *et al.*, J. Biol. Chem. 271:17771-17778, 1996), and thereby transactivates HRE-containing promoters and enhancers. Expression of HIF-1 target genes serves to maintain cellular homeostasis, at least in part, by promoting anaerobic glycolysis, facilitating erythropoiesis, and increasing blood delivery through vasodilatation and angiogenesis (Semenza, Annu. Rev. Cell. Dev. Biol. 15:551-578, 1999). The importance of the HIF-1 response

pathway in human tumorigenesis is underscored by the finding that HIF-1 α is overexpressed in multiple human cancers (Zhong *et al.*, Cancer Res. 59:5830-5835, 1999).

Adaptation to hypoxic conditions not only plays a role in tumor progression, but is also important in conditions of local tissue hypoxia/anoxia, such as in the pathogenesis of myocardial ischemia and stroke. The HIF pathway has also been shown to be important in certain inflammatory conditions, such as Crohn's disease.

Summary of the Invention

The invention provides a method of identifying a compound that modulates a transcriptional response to hypoxia in a cell (*e.g.*, a cultured cell or a cell in an animal). In this method, a cell, or the extracellular environment of a cell, is contacted with a candidate compound. The cell is then subjected to hypoxic conditions, and a transcriptional response of the cell to the hypoxic conditions is assessed. In this method, the transcriptional response can be expression of a reporter gene under the control of a hypoxia-responsive promoter or an endogenous hypoxia-responsive gene. The reporter gene can be, for example, luciferase, green fluorescent protein, yellow fluorescent protein, or cyano-fluorescent protein. The endogenous gene can encode, for example, vascular endothelial growth factor, erythropoietin, heme oxygenase, iNOS, glucose transporter 1, glucose transporter 3, hexokinase, ALDA, or transferrin. The hypoxic conditions to which the cells are exposed can be by exposure to low oxygen levels, or induced chemically, *e.g.*, by deferoxamine or cobalt chloride.

25

The invention also provides a method of identifying a compound that modulates a transcriptional response to hypoxia in a cell. This method involves providing a first transcription factor that is involved in a transcriptional response to hypoxia in a cell, or a fragment thereof; contacting the first
5 transcription factor, or fragment thereof, with a candidate compound; and determining whether the candidate compound affects the interaction of the first transcription factor with a second transcription factor, or a fragment thereof.

Another method provided in the invention is a method of identifying
10 a compound that modulates a transcriptional response to hypoxia in a cell. This method involves providing a first and a second transcription factor that are involved in a transcriptional response to hypoxia in a cell, or fragments thereof; contacting the first and second transcription factors, or fragments thereof, with a candidate compound; and determining whether the candidate compound affects the interaction of the first and second transcription factors.

15 In either of the two methods described immediately above, the first transcription factor can include a peptide of HIF-1 α (*e.g.*, the TAD-C region) and the second transcription factor can include peptide of p300/CBP (*e.g.*, the CH1 region, or, in particular, amino acids 302-418, amino acids 302-443, or amino acids 302-528 of p300/CBP). With both transcription factors, the
20 peptide can include the regions listed above, or larger segments (*e.g.*, entire proteins) including these regions.

The invention also provides a method of treating a condition characterized by hypoxia in a patient, hypoxia in a localized tissue, or activation of the HIF-1 pathway. This method involves administering to a
25 patient a compound that modifies (decreases or increases) a transcriptional response to hypoxia in the patient. In the case of a compound that decreases the response, the method can be used to treat, for example, cancer or an

inflammatory condition of abnormal neovascularization that is mediated by the hypoxia response pathway. In the case of a compound that increases the response, the method can be used to treat, for example, myocardial ischemia, stroke, or an erythropoietin deficiency. The compound can, *e.g.*, bind to HIF-1 α . The invention also includes the use of compounds identified according to the invention in the preparation of medicaments for treating diseases associated with hypoxia, such as those described herein.

Also included in the invention is a method of detecting a hypoxic tissue, cell, or tumor in a patient. This method involves administering to the patient a detectably labeled compound (*e.g.*, a compound that binds to HIF-1 α .) that modifies a transcriptional response to hypoxia in the patient.

The invention provides several advantages. For examples, compounds identified using the methods of the invention can be used as therapeutic molecules for preventing or treating conditions characterized by hypoxia. Thus, compounds that are found to decrease transcriptional responses to hypoxia by, *e.g.*, disrupting the interaction between HIF-1 α and p300/CBP, can be used in methods to treat cancer, resulting in decreased tumor angiogenesis, metabolic derangement, and tumor cell death. Compounds that increase transcriptional responses to hypoxia by, *e.g.*, potentiating the interaction between HIF-1 α and p300/CBP, can be used to treat conditions such as ischemic stroke and myocardial ischemia. Compounds identified according to the invention can also be used to treat inflammatory conditions, such as Crohn's disease, as well as erythropoietin deficiencies (*e.g.*, as found in renal transplant patients). The invention also provides sensitive imaging reagents and methods.

Other features and advantages of the invention will be apparent from the following detailed description and the drawings.

Brief Description of the Drawings

Figure 1A is a schematic representation of HIF-1 α and p300. The amino acid numbers indicated for each protein define the boundaries of the minimal interaction domains.

Figure 1B shows that zinc is required for the interaction of GST-CH1 with HIF-1 α . GST-CH1 (amino acids 302-528) was assayed for its ability to bind to *in vitro* translated (IVT) full length HIF-1 α either in its native state after purification from bacteria or after washing with 1,10-phenanthroline (OP), a heavy metal chelator. The addition of 1 μ M zinc sulfate after chelation restored binding activity.

Figure 1C shows the results of assays of carboxy- and amino-terminal truncation mutants of GST-CH1, with amino acid boundaries as indicated, for their ability to pull down full length IVT HIF-1 α .

Figure 1D shows the results of binding assays of the indicated truncation mutants of the carboxy-terminus of HIF-1 α , constructed as fusions to Gal4 and translated *in vitro*, using GST-CH1 (amino acids 302-443). One fifth of the total IVT product used for each binding assay is indicated (Input), along with the amount bound by GST-CH1 (Bound).

Figure 1E shows the results of assays of the ability of serial substitution mutants of GST-CH1 (amino acids 302-528) produced as GST fusion proteins, with the sequence NAAIRS inserted in place of the indicated amino acids, to bind to IVT full length HIF-1 α .

Figure 2A is a graph showing the results of luciferase and β -galactosidase assays, in which Hep3B cells were cotransfected with an Epo-

luciferase reporter (100 ng), pCMV-LacZ (100 ng), and the indicated expression plasmids (300 ng). Cells were subsequently exposed to either ambient (Normoxia, stippled bars) or 1% (Hypoxia, solid bars) oxygen for 18 hours prior to assay. The results are expressed as corrected relative light units (RLU, mean of three independent transfections \pm S.D.).

Figure 2B is a graph showing the effect of TAD-C expression on p300/CBP dependent transactivation. Hep3B cells were cotransfected with the indicated thioredoxin-constrained fusion protein expression plasmids (300 ng), the indicated Gal4-fusion proteins (20 ng), Gal4-luciferase reporter (100 ng), and pCMV-LacZ (50 ng). Where indicated, cells were exposed to hypoxia for 18 hours. The results are expressed as corrected RLU (mean of three independent transfections \pm S.D.).

Figure 2C shows the results of a ribonuclease protection assay that was conducted to determine VEGF and GAPDH mRNA abundance. In this assay, HepG2 cells were infected at an MOI of 10 with retroviruses encoding Gal4 alone or Gal4 fused with wild type or mutant (MUT) TAD-C polypeptide. Cells were exposed to either ambient or 1% (Hypoxia) oxygen for 18 hours prior to preparation of total RNA.

Figure 2D is a graph showing the levels of cell death in an experiment in which HepG2 cells were infected at an MOI of 5 with retrovirus encoding the indicated Gal4 fusion proteins. Cells were exposed to either ambient or 1% oxygen for 36 hours, and then assayed for cell death by flow cytometric analysis using Annexin V staining and vital dye exclusion. The results are expressed as the mean of three independent experiments \pm S.E.M. The asterisk denotes statistical significance ($p=0.05$) in comparison to controls.

Figure 3A is a graph showing the *in vivo* effect of TADC expression. HCT116 cells were infected at an MOI of 5 with retroviruses encoding Gal4

alone (circle), Gal4 fused with mutant TAD-C polypeptide (Gal4-MUT, square) or with wild type TAD-C polypeptide (triangle). A total of 4×10^6 cells were implanted subcutaneously into nude mice. The results are expressed as the mean \pm S.D. of 8-10 independent tumors. The inset shows an immunoblot for Gal4 in infected cells, demonstrating equivalent levels of expression.

Figure 3B is a graph showing results of the experiments described in reference to Figure 3A for MDA-MB435S cells.

Figure 3C is an immunoblot for p53 in HCT116 cells infected with the indicated retroviruses at an MOI of 10.

Figure 3D is an immunoblot for Gal4, in an experiment in which HCT116 cells were infected with Gal4-MUT or Gal4-TADC at an MOI of 5. Cells were then mixed in equal proportion, and 6×10^6 cells were implanted into nude mice. After three weeks, tumors were excised (Tumor 1-3), and protein extracts were analyzed by immunoblotting for Gal4, along with extracts prepared from cells prior to implantation (Mix).

Figures 4A and 4B are a series of photographs showing that the *in vivo* effect of TAD-C expression can be rescued by a CH1-independent HIF-1 α allele. Tumors arising from HCT116 cells infected with retrovirus encoding Gal4 fused with mutant (MUT) or wild type TAD-C polypeptide at an MOI of 5 were excised after 3 weeks of growth. Paraffin sections were stained with H&E or immunostained for Gal4 or CD31/PECAM. The subcutaneous tissue (SQ), tumor (Tum), and necrotic areas (Nec) are indicated.

Figure 4C is a graph showing the results of experiments in which HCT116 cells were infected with either pBABE-puro-VP16 (VP16) or pBABE-puro-HIF-VP16 (HIFVP), and selected with puromycin. These cells were then infected with retrovirus encoding either Gal4-MUT or Gal4-TADC at an MOI of 5. A total of 6×10^6 cells were implanted in nude mice. Results are the mean of 8

independent tumors \pm S.D.

Detailed Description

Local tissue hypoxia is associated with many different disease states including certain tumors, myocardial ischemia, stroke, certain inflammatory
5 processes, conditions of neovascularization, and certain polycythemic conditions. A multifaceted adaptive response to hypoxia is facilitated by the stabilization and accumulation of the alpha subunit of hypoxia inducible factor 1 (HIF-1). HIF-1 α forms a complex with its binding partner, aryl hydrocarbon
10 receptor nuclear translocator (ARNT), as well as the p300/CBP transcriptional coactivators. This complex induces the transcription of genes that serve to maintain cellular homeostasis in the face of hypoxic conditions. For example, the HIF-1/p300/CBP complex plays a role in inducing expression of genes such as those encoding erythropoietin, which leads to erythropoiesis; vascular
15 endothelial cell growth factor (VEGF), which is a primary mediator of angiogenesis; iNOS and heme oxygenase, which play roles in vasodilation; and the glucose transporter and glycolytic enzymes, which play roles in anaerobic metabolism.

No tractable methods of attenuating the HIF-1 pathway have been previously described. We have now identified a way of disrupting hypoxia-
20 inducible transcription by interfering with the interaction of HIF-1 α with p300/CBP. Specifically, we have identified a region in HIF-1 α that interacts with the CH1 region of p300/CBP, and have shown that disruption of the interaction between these regions can block the transcriptional response to hypoxia in cells. The invention provides methods for identifying reagents or
25 compounds (*e.g.*, small molecules, peptides, proteins, antisense molecules, ribozymes, triple helices, and antibodies, or fragments thereof) that interfere

with the HIF-1 pathway. The disruption can occur at any point in the pathway, for example, at the sensing by the cell of decreased oxygen tension, stabilization and accumulation of HIF-1 α , binding of HIF-1 α to ARNT and p300/CBP, binding of HIF-1 α /ARNT/p300/CBP to hypoxia-responsive elements (HRE) of hypoxia-inducible genes, and transactivation of HRE-associated genes by HIF-1 α /ARNT/p300/CBP. Reagents that block or disrupt this interaction can be used in methods to prevent hypoxia-induced gene expression, and, thus, for example, as anti-neoplastic therapeutics. Such reagents can also be used to treat inflammatory diseases, such as Crohn's disease, retinopathies, and polycythemia. Reagents that potentiate this interaction can be used, for example, in methods of treating ischemic stroke or myocardial ischemia.

Reagents that modify transcriptional responses to hypoxia, *e.g.*, through disrupting the HIF-1 α /p300/CBP CH1 interaction that is involved in cellular responses to hypoxia, can be identified using any of a number of standard methods, including *in vitro*, cell-based, and *in vivo* methods. Of particular interest are high throughput screening methods employing, for example, libraries contained on chips. Such methods are well known in the art, and can readily be adapted for use in the invention.

Specific examples of screening methods that can be used in the invention are as follows. For example, a fusion protein, such as a GST fusion protein, containing the portion p300/CBP that binds to HIF-1 α (*i.e.*, a peptide including the p300/CBP CH1 region (*e.g.*, p300/CBP 302-418, p300/CBP 302-443, p300/CBP 302-528, or any fragment including amino acids 302-418, up to full length p300/CBP; see below)) can be produced in a bacterial system. A labeled (*e.g.*, S³⁵-labeled), *in vitro* transcription/translation HIF-1 α peptide (*e.g.*, TAD-C or a peptide or polypeptide including TAD-C, up to full length

HIF-1 α ; see below) can then be assayed for binding to the fusion protein. In a variation of this method, the HIF-1 α peptide is made as a fusion protein, and binding of a labeled p300/CBP CH1 region-containing peptide is measured. Details of such an example of an *in vitro* binding assay that can be used in the invention are provided below (see Experimental Results and Materials and Methods, below).

An additional example of an *in vitro* method that can be used in the invention is an ELISA-based assay, in which GST-CH1 is expressed and purified from bacteria and a HIF polypeptide (*e.g.*, a HIF peptide containing amino acids 786-826) is synthesized as a biotinylated synthetic peptide. The ELISA is set up by immobilizing the biotinylated HIF peptide on a streptavidin coated plate. Candidate compounds are then added and, subsequently, GST-CH1. After washing, the amount of bound GST-CH1 is assayed by using an enzyme-linked anti-GST antibody. The assay can be varied, for example, by immobilizing GST-CH1, adding the biotinylated HIF peptide, and detecting binding with enzyme linked strepavidin. Also, CH1 and the HIF polypeptides can be reversed in their roles in the assays described above, or could be alternatively tagged with other epitopes (*e.g.*, HA, myc, or HIS6), and activity could be measured by enzymes linked to the appropriate antibody. Moreover, different binding partners in the pathway of hypoxia-induced transcription can be used in the methods described above. For example, HIF-1 α and ARNT, or HIF and the hypoxia-response element of hypoxia-induced genes, can be used as target binding partners in these methods. Additional *in vitro* binding methods that can be used in the invention are known to those of skill in this art. The methods are carried out in the presence and absence of a candidate compound, and detection of a change in binding can be used as a read out for the identification of a compound that may be useful in modulating the cellular

response to hypoxia in the invention. As is mentioned above, compounds that are found to disrupt or interfere with binding are candidates for treating conditions in which it is desirable to block the hypoxic response (*e.g.*, neoplastic diseases; see above), while compounds that are found to increase binding are candidates for treating conditions in which it is desirable to potentiate the hypoxic response (*e.g.*, myocardial ischemia and stroke). In addition, because HIF-1 is the primary regulator of erythropoietin production, compounds found to increase transcriptional responses to hypoxia may have utility in treating patients having conditions associated with a deficiency of erythropoietin, *e.g.*, renal transplant patients and patients with anemia, of any etiology.

In addition to *in vitro* methods, such as those described above, any of a number of cell-based methods can be used to identify or characterize candidate compounds according to the invention. For example, cells can be subjected to hypoxic conditions in the presence or absence of a candidate compound, and differences in the hypoxic phenotype can be detected. For example, expression of a hypoxia-induced gene, such as VEGF or erythropoietin, can be measured (*e.g.*, by RNase protection, Northern blot analysis, quantitative RT-PCR, or real-time PCR), or cell death can be measured. Alternatively, cells can be transfected with a construct including a reporter gene (*e.g.*, a luciferase, fluorescent protein, or β -galactosidase gene) and a hypoxia-responsive promoter (*e.g.*, the erythropoietin promoter), and the effect of the compound on expression of the reporter gene can be measured. Additional cell-based methods are known to those of skill in this art, and use of these methods is included in the invention.

For example, a cell-based assay can be used to identify compounds that act at different points along the HIF pathway. Such an assay can involve use of a cell line having a stably integrated hypoxia-inducible reporter gene (*e.g.*, luciferase, a fluorescent protein, or β -galactosidase gene) under the control of a hypoxia responsive promoter or enhancer element (*e.g.*, VEGF or erythropoietin). Cells are plated in a multiwell format, candidate compounds are added, and then the cells are made hypoxic either by true hypoxia (*e.g.*, 1% oxygen) or by adding deferoxamine or CoCl_2 to artificially activate the HIF pathway. Hypoxia-inducible transcription can then be read out by fluorescence or luciferase activity. In a variation of this method, a second fluorescent protein or Renilla luciferase can, in the same cell, be constitutively expressed under the control of another promoter as an internal standard. This allows control for non-specific suppression of transcription or non-specific cytotoxicity, which would result in decreased fluorescence of the constitutive marker, as well as the hypoxia-responsive marker. Again, this method has the advantage of identifying compounds that target p300/HIF-1 α , but also can be used to identify compounds that target other nodes on the pathway (*e.g.*, the ARNT/HIF-1 α interaction, the HIF-1/DNA interaction, and HIF-1 α stabilization). This assay could also identify compounds that up-regulate the hypoxia pathway. Such compounds may have utility in terms of being pro-angiogenic (*e.g.*, for treating coronary artery disease, to increase EPO production, etc.).

For any two specific targets, a mammalian two hybrid-based assay can also be used. For example, one partner can be expressed fused to yellow fluorescent protein (*e.g.*, YFP-CH1) and the other partner fused to cyanofluorescent protein (*e.g.*, CFP-HIF-1 α). Cells are stably transfected to express these fusion proteins. The interaction of the proteins can be scored by FRET

(fluorescence resonance energy transfer). Compounds that result in loss of FRET are identified as disrupting the protein-protein interaction. Alternatively, a cell can be engineered to express Gal4-CH1, HIF-VP16, and a Gal4-GFP reporter. Loss of GFP signal indicates identification of a compound that
5 disrupts the mammalian two hybrid interaction between Gal4-CH1 and HIF-VP16, with resulting decreased GFP expression off of the ectopic Gal4 promoter. These assays, relying on ectopically expressed proteins, do not necessarily require hypoxic conditions.

Another example of a cell-based assay involves linking a hypoxia-
10 responsive element (HRE) to a potentially toxic gene product (*e.g.*, HRE-TK), and then selecting for compounds that prevent cytotoxicity (*e.g.*, with gancyclovir) under hypoxic conditions (real or chemically induced). Such an assay scores positive for compounds that inhibit hypoxia-inducible transcription and are in and of themselves not toxic.

15 All of the assays described above can be set up as high-throughput screens of libraries of natural and synthetic compounds. For example, libraries of compounds, such as peptides or proteins, that are immobilized on chips can be used. In addition, the cell-based assays are particularly amenable for use in screening for polypeptides or proteins that inhibit the hypoxia-response.
20 Specifically, high titer/high complexity retroviral libraries have been constructed encoding for polypeptides, anti-sense RNA, or random peptides. Such retroviral libraries can be used to infect a stably integrated HRE-reporter cell and, by fluorescence-activated cell sorting, cells in which the hypoxia-response has been changed, for example, diminished or increased (*e.g.*, as
25 detected by changes in fluorescence), can be sorted out at a frequency of 1/100,000-1/1,000,000. The sequence encoded by the integrated retrovirus can be determined by rescuing the insert using PCR. In addition, crystal or solution

structures of components of the HIF pathway, fragments of these components, such as HIF polypeptides (*e.g.*, TAD-C), p300/CBP (*e.g.*, CH1), as well as p35srj, or complexes of these components, can be used in the identification of small molecules that mimic the 3D structures, using standard databases.

5 In addition to *in vitro* and cell-based methods, such as those described above, *in vivo* methods can be used to identify or characterize compounds according to the invention. One example of an *in vivo* method is a tumor implantation model, involving xenografts into nude mice. In such a method, tumor cells are implanted into nude mice, which are then either treated with a
10 candidate compound or left untreated. The effect of a candidate compound can be determined, for example, by measuring tumor size or by postmortem histological analysis (*e.g.*, analysis of levels of HIF-1 α , VEGF, angiogenesis, and cell death). Additional tumor models are known in the art and can be used in the methods of the invention. As compounds become characterized as
15 having a desired effect (*e.g.*, decreasing tumor growth or angiogenesis), they can be analyzed for effects on models of specific types of cancers, in an effort to determine which types of human diseases may be treated using the compounds. *In vivo* methods can also be used to identify or characterize compounds that potentiate hypoxia-induced transcription, and thus are
20 candidate compounds for treating myocardial ischemia or stroke.

 In an additional *in vivo* method, tumor cells can be engineered to express luciferase, such cells can be implanted into mice, and luciferase activity can be detected in the live animal after tail vein injection of luciferin. Specifically, utilizing a sensitive CCD camera, photons can traverse through the skin of the
25 animal and light emission can be detected and quantified in a non-invasive way. Animals can be followed longitudinally through time as this is a non-lethal technique. As an extension of such technology, we have engineered tumor cell

lines that have luciferase driven by a hypoxia-responsive element (multimerized Epo enhancer HRE). Once injected into nude mice, the amount of tumor hypoxia can be quantified as tumors enlarge. At a given tumor volume, after quantifying hypoxia-inducible luciferase activity, compounds of interest can be
5 injected. The hypoxia-specific reporter activity in tumor cells can then be serially followed, in the same animal, as treatment with the compound is continued. Any compound that specifically suppresses hypoxia-inducible transcription *in vitro* can thus be assayed for the ability to do so *in vivo* in an intact animal. Transgenic mice with a HRE-luciferase integrated in every cell
10 have been made. Using such an animal, we can determine the effect of a given compound on any "normally" occurring local tissue hypoxia (*e.g.*, whether there is any basal hypoxia-inducible transcription in highly oxygen dependent organs, such as brain and kidneys, and how the candidate compounds effect this activity). As a further extension, HRE-Renilla luciferase integrated tumor cell
15 lines can be used. Such tumor cell lines are implanted into HRE-luciferase transgenic mice, so that tumor cell hypoxia and host animal hypoxia can be separately measured. Such a system can be used to titrate compound doses to achieve a therapeutic index, in which tumor cell hypoxia-inducible transcription was reduced, but not normal tissue activity.

20 In addition to the screening methods described above, standard methods for preparing peptide mimetics can be carried out to obtain molecules that modify transcriptional responses to hypoxia, *e.g.*, by disrupting the interaction between HIF-1 α and p300/CBP. In addition to small molecule modifiers that affect, *e.g.*, the interaction between HIF-1 α and CH1, peptide molecules can be
25 identified. For example, phage display can be used to optimize or identify peptide ligands of the CH1 and HIF-1 α binding domains. Compounds identified using these methods can be tested using the *in vitro*, cell-based, and

in vivo systems described above.

p35srj is a protein that has a negative regulatory function on the HIF-1 pathway (Bhattacharya *et al.*, Genes Dev. 13(1):64-75, 1999). It interferes with this pathway by binding to p300/CBP and inhibits HIF-1 transactivation by
5 blocking the HIF-1 α /p300 CH1 interaction. P35srj can thus be used in the invention as a prototype protein that interferes with the HIF-1 pathway.

Compounds identified using the methods of the invention can be administered to patients in need of treatment (*e.g.*, patients having cancer, any of the other diseases listed above, or any other disease or condition associated
10 with hypoxia) using standard modes of administration, such as by oral, intravenous, subcutaneous, intramuscular, and parenteral administration. Appropriate dosages of compounds identified using the methods of the invention can be readily determined by one of skill in the art, and can be, for example, 0.001-50 mg/kg/patient. In addition, peptide-based compounds can
15 be administered using gene therapy methods.

Compounds identified using the methods of the invention can also be used in imaging methods. As is discussed above, HIF-1 α is unstable under normoxic conditions, and thus normal cells do not contain detectable levels of HIF-1 α . Compounds that are identified as binding to HIF-1 α , according to the
20 invention, thus can be labeled for use in high resolution imaging methods to detect cancer cells and tumors, as well as ischemic tissue resulting from, *e.g.*, stroke or heart disease.

The methods of the invention are based, in part, on the following experimental results.

25 Experimental Results

The detailed structural requirements for p300/CBP binding to HIF-1 α

were mapped by *in vitro* assays utilizing a series of GST-fused deletion mutants centered around the CH1 domain of p300, the primary HIF-1 α interacting unit of that protein (Arany *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:12969-12973, 1996; Kallio *et al.*, EMBO J. 17:6573-6586, 1998). These mutant fusion
5 proteins were assayed for binding to full length, *in vitro* translated HIF-1 α , a zinc-dependent process (Figure 1B). The minimal HIF-1 α binding domain proved to encompass 116 residues (amino acids 302-418 of p300), which includes the CH1 domain and some flanking sequences (Figures 1A and 1C). To map the minimal p300-binding domain of HIF-1 α , a series of Gal4-fused
10 truncation mutants of the C-terminus were generated. These Gal4 fusion proteins were translated *in vitro* and probed for their ability to bind to GST-CH1 (amino acids 302-443). The C-terminal 41 residues of HIF-1 α , the so called C-terminal transactivation (TAD-C) domain (Jiang *et al.*, J. Biol. Chem. 272:19253-19260, 1997) were found to be necessary and sufficient for binding
15 to GST-CH1 (Figures 1A and 1D).

To study further the requirements for HIF-1 α /CH1 binding, a series of GST-CH1 (amino acids 302-528) internal substitution mutants were generated. These serial, non-overlapping mutants spanned residues 341-424 of p300. In each mutant, five residues were deleted and the sequence, Asp-Ala-Ala-Ile-
20 Arg-Ser (NAAIRS), inserted. The NAAIRS sequence can assume an α -helical or β -strand conformation (Wilson *et al.*, Proc. Natl. Acad. Sci. U.S.A. 82:5255-5259, 1985), thus, decreasing the likelihood of generalized conformational disruption following the deletion/insertion event. Almost all substitutions abolished CH1 binding to full length HIF-1 α (Figure 1E), suggesting that the
25 CH1 domain must assume a complex, zinc-coordinated conformation in order to interact with HIF-1 α .

Overproduction of polypeptides corresponding to these minimal binding

domains (TAD-C or CH1) led to attenuation of hypoxia-inducible reporter activity (Figure 2A). Moreover, only those TAD-C and CH1 mutants that bound to an intact target *in vitro* (Figures 1C and 1D) had a dominant-negative effect on hypoxia-inducible reporter activity *in vivo* (Figure 2A). In titration experiments, it appeared that TAD-C polypeptides were more potent suppressors of HIF function than were CH1-containing dominant-negative polypeptides, perhaps because of the imputed conformational complexity of CH1. Accordingly, subsequent experiments focused on the activity of a wild type TAD-C polypeptide (amino acids 786-826 of HIF-1 α) compared to that of an eight residue truncated mutant (amino acids 786-818 of HIF-1 α , which was unable to bind to GST-CH1 (Figure 1D) and which failed to exert a dominant-negative effect on hypoxia-inducible reporter activity (Figure 2A).

By comparison with its effects on HIF-1 α -mediated transcriptional activity, TAD-C polypeptide overexpression had no effect on RAR, SREBP2, or SRC-1, which interact with the N-terminal (Chakravarti *et al.*, Nature 383:99-103, 1996), KIX (Oliner *et al.*, Genes Dev. 10:2903-2911, 1996) and the glutamine-rich (Yao *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:10626-10631, 1996) domains of p300/CBP respectively (Figure 2B). However, it did inhibit the transcriptional activity of STAT2, which, like HIF-1 α , uses CH1 to mediate its coactivation (Arany *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:12969-12973, 1996; Bhattacharya *et al.*, Nature 383:344-347, 1996). These results imply that an intact TAD-C polypeptide binds and blocks access of multiple proteins to the CH1 domain, without inducing a generalized suppressive effect on other p300/CBP functions.

To facilitate expression of the relevant TAD-C and mutant polypeptides, VSV-G pseudotyped retroviruses were engineered to express Gal4 carrier protein alone or Gal4 fused to either wild type or mutant TAD-C polypeptide. HepG2 cells were infected with these retroviruses at a multiplicity of infection (MOI) of 10, exposed to either normal or hypoxic conditions, and VEGF mRNA levels were determined by a ribonuclease protection assay. Equivalent expression of Gal4 and the Gal4 fused polypeptides was regularly achieved in this and related experiments (for example, see Figure 3A, inset). Concordant with the reporter assays, noted above, VEGF mRNA abundance was reproducibly diminished in cells expressing wild type but not mutant TAD-C polypeptide, both under normoxic as well as hypoxic conditions (Figure 2C). The overall abundance of HIF-1 α under hypoxic conditions was not altered by introduction of TAD-C.

Cell survival under normoxic conditions was essentially the same in HepG2 cells expressing either Gal4 alone or Gal4 fused to TAD-C or mutant polypeptide (Figure 2D). Cell growth was also not affected under normoxic conditions. Under hypoxic conditions, however, cell death was slightly, but significantly, increased in cells expressing wild type TAD-C polypeptide, in comparison to cells expressing Gal4 alone or Gal4 fused with mutant polypeptide (Figure 2D).

To extend these findings, the growth of modified human tumor cells was assayed in a nude mouse xenograft model. Tumor cells were retrovirally transduced at an equal MOI, resulting in equivalent expression of Gal4, or Gal4 fused to wild type or mutant TAD-C polypeptide (Figure 3A, inset). Tumor growth was significantly and reproducibly attenuated in HCT116 (Figure 3A) or MDA-MB435S (Figure 3B) cells expressing wild type TAD-C polypeptide when compared to the same cells expressing Gal4 alone or Gal4 fused to

mutant polypeptide (Figures 3A and 3B). This effect was independent of p53 status, since HCT116 cells have wild type p53, while MDA-MD435S cells express mutant p53. Furthermore, immunoblot analysis for p53 demonstrated no difference in p53 abundance in HCT116 cells expressing these three ectopic
5 Gal4 polypeptides (Figure 3C).

We were interested in learning whether the anti-tumor effect of TAD-C polypeptide expression was due to a paracrine effect, where a cell producing TAD-C elaborates a factor(s) which suppresses growth of surrounding tumor cells. Specifically, tumor cells expressing either Gal4-TAD-C or Gal4-mutant
10 polypeptide were mixed in equal numbers prior to implantation. When tumors were excised after three weeks of growth, there was a strong bias towards cells expressing the mutant polypeptide (Figure 3D), suggesting that the anti-tumor effect of expressing TAD-C was not a result of a paracrine effect arising from the elaboration of a trans-dominant anti-tumor factor.

15 Necropsy and histological examination of the injection site of tumor cells engineered to express Gal4 fused TAD-C polypeptide revealed large areas of central necrosis with a small rim of viable tumor cells adjacent to the subcutaneous tissue (Figure 4A), even in the absence of grossly measurable tumor at these sites (Figure 4B). Control tumors arising from cells expressing
20 Gal4 or Gal4 fused to mutant polypeptide revealed small areas of necrosis, but vast areas of viable tumor (Figure 4A) were grossly palpable (Figure 4B). Immunostaining for the endothelial cell marker, CD31/PECAM, revealed abundant capillary formation within control tumor segments compared to TAD-C expressing tumors (Figure 4A). It is unclear whether this was a primary or
25 secondary effect, *i.e.*, that there were fewer tumor cells because of diminished vessel density, or vice versa.

Since several, known transcription factors interact with the CH1 domain of p300, and TAD-C polypeptide expression appears to generally disrupt CH1 function (Figure 2B), we asked whether interference with HIF-1 α binding/function is a critical component of the TAD-C anti-tumor effect.

- 5 Accordingly, a HIF-1 α allele was engineered in which the oxygen-degradation domain was deleted, and TAD-C was replaced with VP16 (HIF-VP16), creating a constitutively active, CH1-independent HIF-1 α molecule. HCT116 cells were first transduced with either VP16 alone or with HIF-VP16. After selection, these cells were transduced with Gal4 fused to wild type or mutant
- 10 TAD-C polypeptide. With coexpression of unfused VP16, tumor growth was attenuated by TAD-C in comparison to the mutant polypeptide (Figure 5B), in agreement with the results summarized above. However, with coexpression of HIF-VP16, there was identical tumor growth by cells coexpressing either TAD-C or mutant polypeptide (Figure 5B). By immunoblot analysis, coexpression of
- 15 HIF-VP16 had no effect on expression of TAD-C or mutant polypeptide in comparison to coexpression of VP16 alone. Thus, other effects notwithstanding, disrupting the interaction of HIF-1 α with CH1 is a necessary event in TAD-C mediated tumor growth suppression.

- The experimental results described above were obtained using the
- 20 following materials and methods.

Materials and Methods

- Plasmids and Retroviruses.* Plasmids were generated using standard techniques (Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Wiley-Interscience, New York, 1988)) and were verified by sequencing. All
- 25 mammalian expression vectors utilized CMV promoters. GST fusion proteins were generated by subcloning PCR fragments into pGEX vectors (Pharmacia).

Gal4-fusion proteins were made in pCMX-Gal4(N) (R. Evans). Scanning NAAIRS mutagenesis of GST-CH1(amino acids 302-528) was performed using single strand mutagenesis (Muta-Gene, Bio-Rad) according to manufacturer's instructions. pCMV-CH1 mutants were constructed by

5 subcloning PCR products in pCMV/myc/nuc (Invitrogen). A thioredoxin-constrained expression vector was created by subcloning PCR fragments of *E. coli* thioredoxin A into pcDNA3.1 (Invitrogen), creating new unique restrictions sites within the active loop similar to prior reports (LaVallie *et al.*, Biotechnology (NY) 11:187-193, 1993). The HIF-1 α plasmid used for *in vitro*

10 translation, Epo-luciferase reporter, Gal4-luciferase reporter, and pCMV-LacZ, Gal4-SRC1, Gal4-SREBP2, Gal4-RAR, Gal4-STAT2, and Gal4-HIF1 α expression vectors have been described previously (Arany *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:12969-12973, 1996; Yao *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:10626-10631, 1996; Bhattacharya *et al.*, Nature 383:344-347, 1996;

15 Bhattacharya *et al.*, Genes Dev. 13:64-75, 1999). VSV-G pseudotyped retroviruses were constructed in pMMP, and packaged by cotransfection with pMD-MLV and pMD-G (R. Mulligan) in 293T cells. Titers were determined by infection of the intended target cell line. HIF-VP16 and VP16 retroviruses were constructed in pBABE-puro, and packaged and titered, as noted above.

20 *In vitro translation and in vitro protein binding assays.* Except where noted, all reagents were from Sigma. *In vitro* transcription/translation (IVT) was performed in the presence of ³⁵S-methionine (NEN) using T3 or T7 polymerase coupled with rabbit reticulocyte lysates (TNT, Promega) according to the manufacturer's instructions. GST fusion proteins were produced in, and

25 purified from, bacteria, and binding of IVT proteins was performed as previously described (Grossman *et al.*, Mol. Cell. 2:405-415, 1998). Except where indicated, binding and wash buffer consisted of 20 mM Tris-HCl (pH

8.0), 150 mM NaCl, 0.2% NP-40, 1 mM DTT, 10 μ M ZnCl₂, 2 μ M EDTA, PMSF 50 μ g/ml, pepstatin 1 μ g/ml, leupeptin 1 μ g/ml, and aprotinin 1.9 μ g/ml. Where indicated, heavy metals were chelated by three changes of wash buffer containing 1,10-phenanthroline (Aldrich) at 10 mM. Zinc reconstitution after
5 chelation of heavy metals utilized ultrapure zinc chloride (>99.999%, Aldrich).

Reporter assays. Luciferase and β -galactosidase assays were performed as previously described (Bhattacharya *et al.*, Genes Dev. 13:64-75, 1999). In all cases, luciferase activity was corrected for β -galactosidase activity as an internal control for transfection efficiency and global changes in transcriptional
10 activity. Results are expressed as corrected relative light units (RLU). For each reporter experiment, at least two independent experiments were performed.

Hypoxia. Hypoxia was achieved by incubating cells in a humidified environment at 37°C in a Cellstar three gas incubator (Harris) maintained at
15 10% CO₂ and 1% O₂. Accurate O₂ and CO₂ regulation was periodically verified with a Fryrite gas analyzer (Bacharach).

Cell culture and retroviral infection. Tumor cell lines were obtained from the ATCC, and were cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and L-glutamine (Gibco-BRL). Cells in logarithmic
20 growth phase were serially infected 12 hours apart with VSV-G pseudotyped pMMP-Gal4 retroviruses to achieve a final MOI of 5 or 10, depending on the experiment. No selection was used, since immunostaining routinely demonstrated 100% infection of cells at these MOI. For pBABE-puro-VP16 or pBABE-puro-HIF-VP16, cells were infected at an MOI of <1 and selected with
25 puromycin (2 μ g/ml) for 4 days. Cells were allowed to recover for 1 day and then infected at an MOI of 5 with pMMP-Gal4 retroviruses.

Immunoblot, ribonuclease protection, and cell death assays.

Immunoblot analyses for Gal4 (RK5C1, Santa Cruz Biotechnology), p53 (DO-1, Oncogene Research), and HIF-1 α (OZ15 (Arany *et al.*, Proc. Natl. Acad. Sci. U.S.A. 93:12969-12973, 1996)) were performed using standard techniques (Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Wiley-Interscience, New York, 1988)). Total RNA was isolated (RNeasy, Qiagen), and ribonuclease protection assays performed utilizing a multiprobe human angiogenesis kit (RiboQuant, Pharmingen) according to the manufacturer's instructions. Cell death was measured by staining with anti-Annexin V antibody (Pharmingen) and 7-amino-actinomycin D (Pharmingen), and analyzed by flow cytometry according to the manufacturer's instructions.

Xenograft tumor model. Nude mice (Taconic) were obtained at 6-8 weeks of age and allowed to acclimate for 1 week prior to tumor implantation. Tumor cells were allowed to recover for at least 48 hours after retroviral infection prior to implantation into nude mice. The indicated number of tumor cells was injected subcutaneously and bilaterally into the flanks of mice. Tumors were measured with calipers, and tumor volume was calculated as $0.5 \times L \times W^2$. For each cell line, at least two independent experiments were performed. Where indicated, tumors were excised and fixed in 4% paraformaldehyde in phosphate buffered saline or minced and lysed in RIPA/300 buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 1 mM DTT, protease inhibitors). Immunostaining with anti-Gal4 (IKE, Santa Cruz Biotechnology) or anti-CD31 (MEC13.3, Pharmingen) was performed using the Vectastain ABC Elite system (Vector) according to the manufacturer's instructions.

All references cited herein are incorporated by reference. Other embodiments are within the following claims.

What is claimed is:

1. A method of identifying a compound that modulates a transcriptional response to hypoxia in a cell, said method comprising:

contacting a cell, or the extracellular environment of a cell, with a candidate compound;

5 subjecting the cell to hypoxic conditions; and
assessing a transcriptional response of the cell to the hypoxic conditions.

2. The method of claim 1, wherein the transcriptional response is expression of a reporter gene under the control of a hypoxia-responsive promoter or an endogenous hypoxia-responsive gene.

10 3. The method of claim 2, wherein the reporter gene encodes luciferase, green fluorescent protein, yellow fluorescent protein, or cyano-fluorescent protein.

4. The method of claim 2, wherein the endogenous gene encodes vascular endothelial growth factor, erythropoietin, heme oxygenase, iNOS,
15 glucose transporter 1, glucose transporter 3, hexokinase, ALDA, or transferrin.

5. The method of claim 1, wherein the cell is a cultured cell.

6. The method of claim 1, wherein the cell is in an animal

7. The method of claim 1, wherein the hypoxic conditions to which the cells are exposed induced by deferoxamine or cobalt chloride.

8. A method of identifying a compound that modulates a transcriptional response to hypoxia in a cell, said method comprising:

providing a first transcription factor that is involved in a transcriptional response to hypoxia in a cell, or a fragment thereof;

5 contacting said first transcription factor, or fragment thereof, with a candidate compound; and

determining whether the candidate compound affects the interaction of the first transcription factor with a second transcription factor, or a fragment thereof.

10 9. A method of identifying a compound that modulates a transcriptional response to hypoxia in a cell, said method comprising:

providing a first and a second transcription factor that are involved in a transcriptional response to hypoxia in a cell, or fragments thereof;

15 contacting the first and second transcription factors, or fragments thereof, with a candidate compound; and

determining whether the candidate compound affects the interaction of the first and second transcription factors.

20 10. The method of claim 8 or 9, wherein said first transcription factor comprises a peptide of HIF-1 α and said second transcription factor comprises a peptide of p300/CBP.

11. The method of claim 10, wherein said peptide of HIF-1 α comprises the TAD-C region of HIF-1 α .

12. The method of claim 11, wherein said peptide of HIF-1 α consists essentially of the TAD-C region of HIF-1 α .

13. The method of claim 10, wherein said peptide of p300/CBP comprises the CH1 region of p300/CBP.

14. The method of claim 13, wherein said peptide of p300/CBP comprises amino acids 302-418, amino acids 302-443, or amino acids 302-528
5 of p300/CBP.

15. The method of claim 13, wherein said peptide of p300/CBP consists essentially of the CH1 region of p300/CBP.

16. A method of treating a condition characterized by hypoxia in a patient, hypoxia in a localized tissue, or activation of the HIF-1 pathway, said
10 method comprising administering to the patient a compound that modifies a transcriptional response to hypoxia in the patient.

17. The method of claim 16, wherein the compound decreases a transcriptional response to hypoxia in the patient.

18. The method of claim 17, wherein the condition is cancer or an
15 inflammatory condition of abnormal neovascularization that is mediated by the hypoxia response pathway.

19. The method of claim 16, wherein the compound increases a transcriptional response to hypoxia in the patient.

20. The method of claim 19, wherein the condition is myocardial
20 ischemia, stroke, or an erythropoietin deficiency.

21. The method of claim 16, wherein the compound binds to HIF-1 α .

22. A method of detecting a hypoxic tissue, cell, or tumor in a patient, said method comprising administering to the patient a detectably labeled compound that modifies a transcriptional response to hypoxia in the patient.

5 23. The method of claim 22, wherein the compound binds to HIF-1 α .

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(54) Title: IDENTIFICATION OF COMPOUNDS THAT MODIFY TRANSCRIPTIONAL RESPONSES TO HYPOXIA

(57) Abstract: The invention provides methods for identifying compounds that can be used to modify transcriptional responses to hypoxia, and use of such compounds in therapeutic and diagnostic methods.

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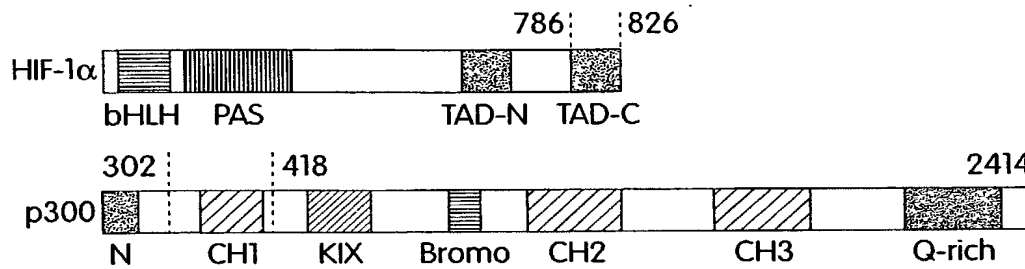


Fig. 1a

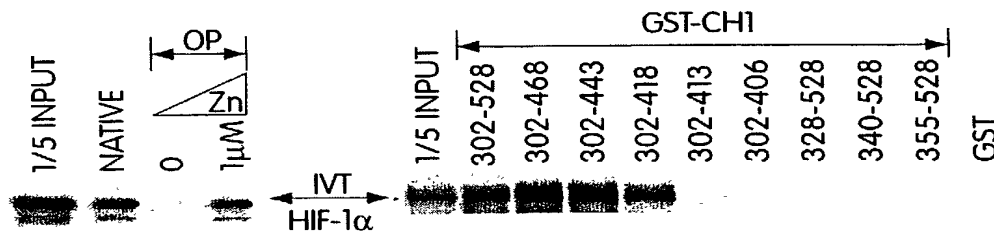


Fig. 1b

Fig. 1c

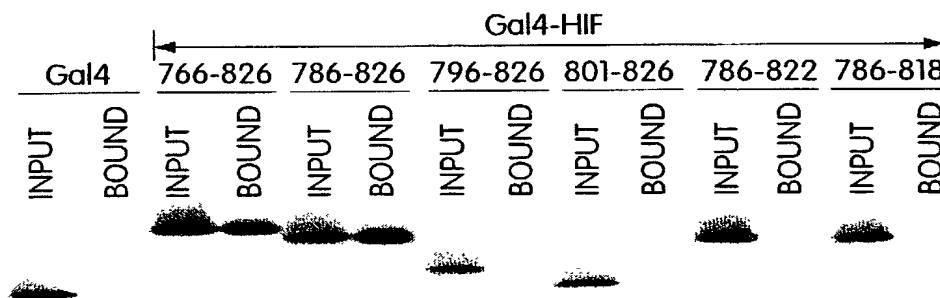


Fig. 1d

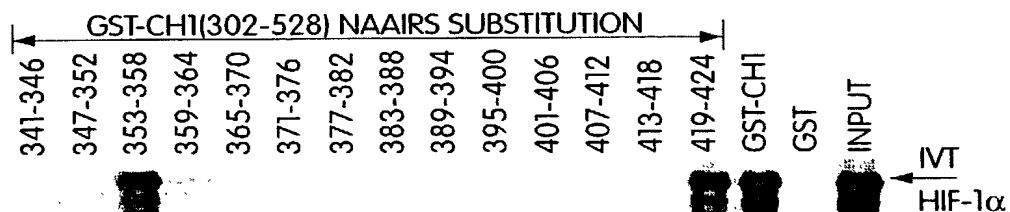


Fig. 1e

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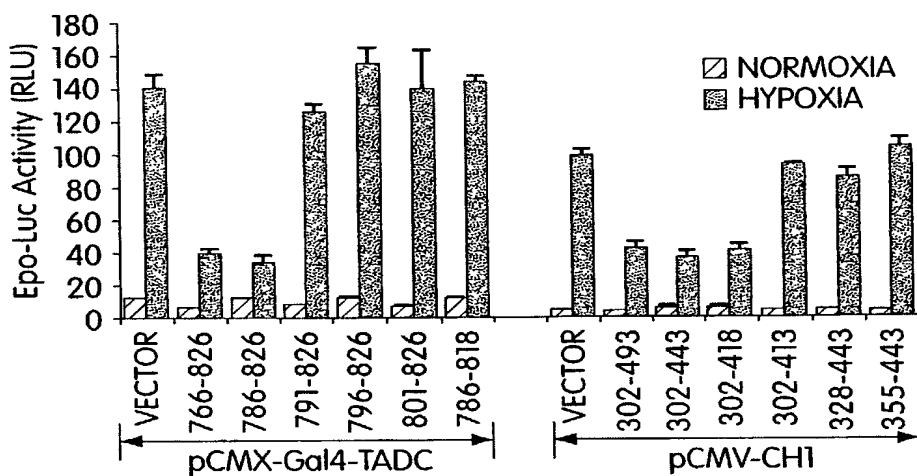


Fig. 2a

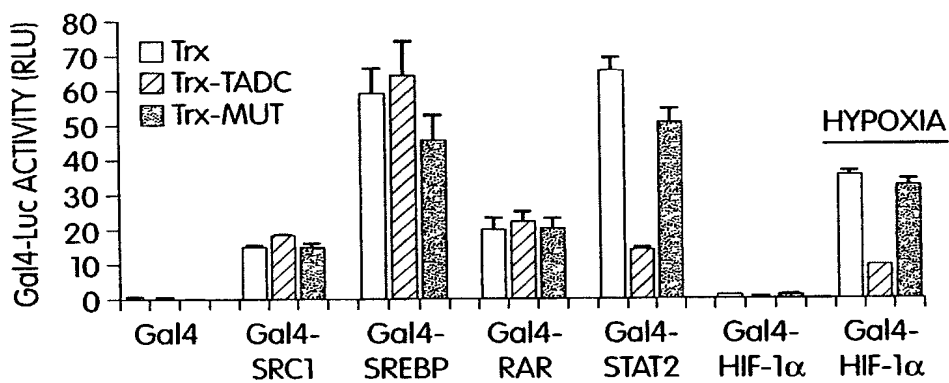


Fig. 2b

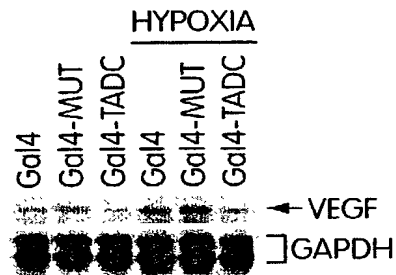


Fig. 2c

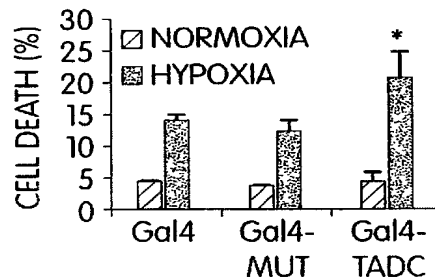


Fig. 2d

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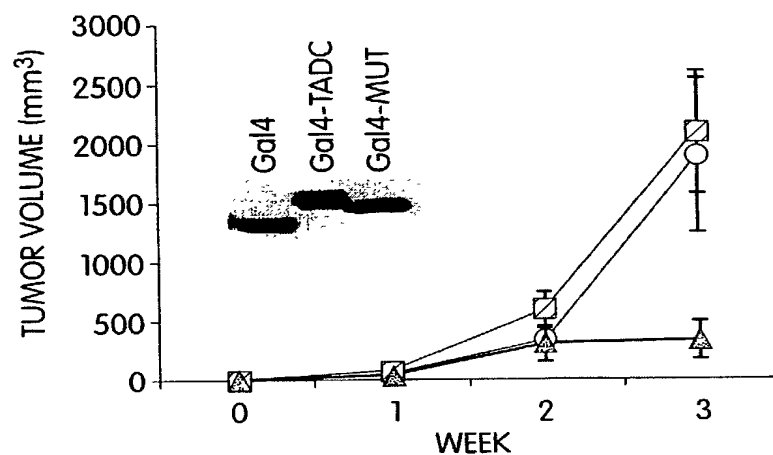


Fig. 3a

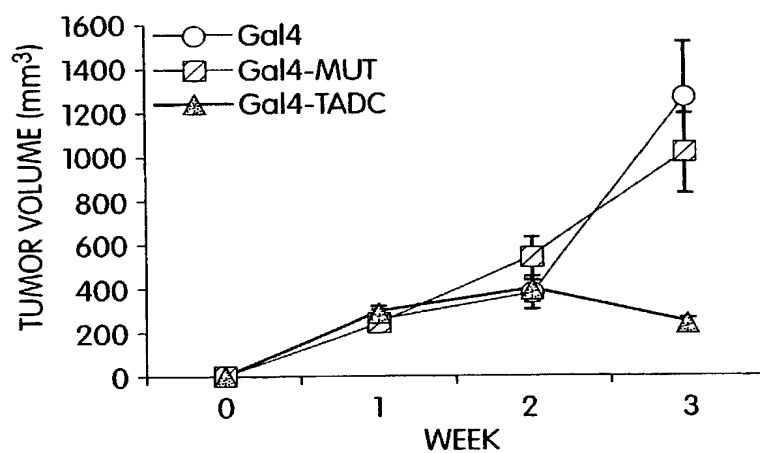


Fig. 3b

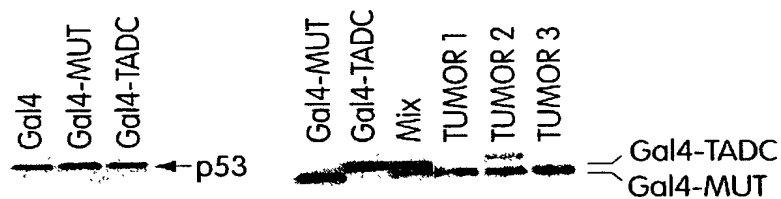


Fig. 3c

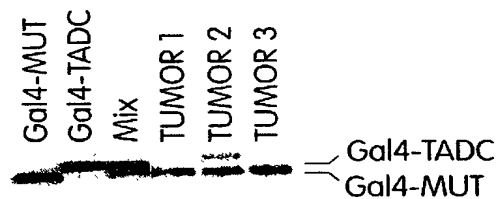


Fig. 3d

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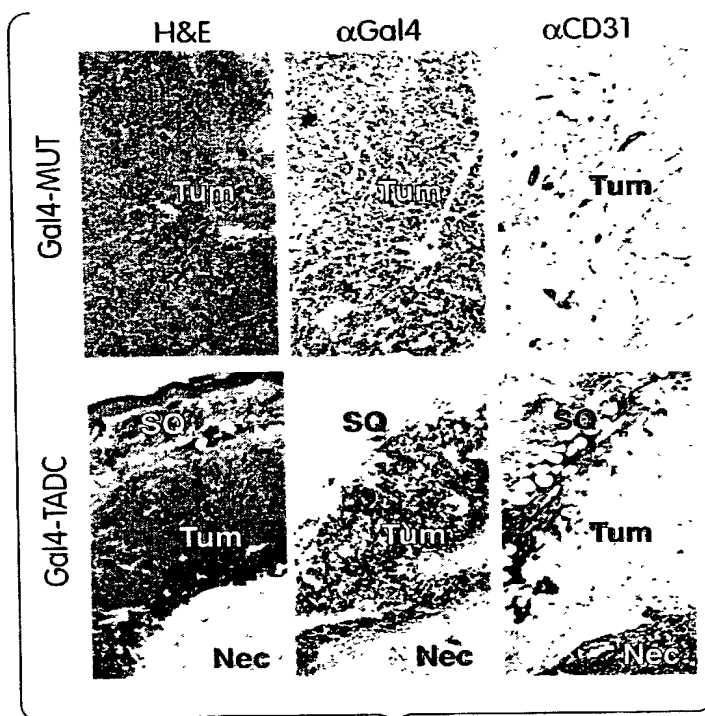


Fig. 4a

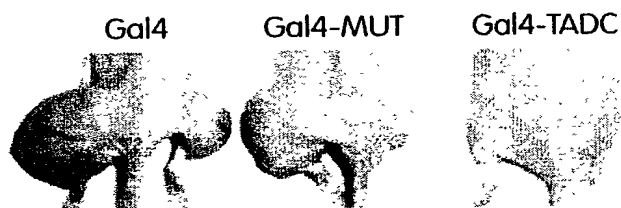


Fig. 4b

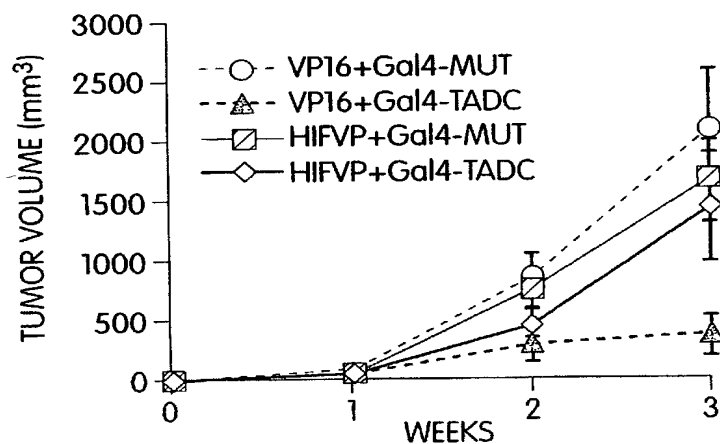


Fig. 4c

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20020515 09:03:00
PATENT APPLICATION
Attorney Docket No. 20363-012 NATL

**COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a utility patent is sought on the invention entitled:

**IDENTIFICATION OF COMPOUNDS THAT MODIFY TRANSCRIPTIONAL
RESPONSES TO HYPOXIA**

the specification of which was filed in the United States Patent and Trademark Office on **December 4, 2001**, as United States non-provisional Application No. **10/009,584**, and bearing Attorney Docket No. **20363-012 NATL**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

- ☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Appln. Number	Country (if PCT, so indicate)	Filing Date (dd/mm/yy)	Priority Claimed	
			Yes	No

- ☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing

Applicant: Livingston, et al
Application No.: 10/009,584

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PCT International Applications designating the United States:

PCT International Application No.	PCT Filing Date	Status
US00/15325	June 2, 2000	Published

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Attorney or Agent	Registration No.	Attorney or Agent	Registration No.
Kevin Ainsworth	<u>39,586</u>	Shane Hunter	<u>41,858</u>
Ingrid Beattie	<u>42,306</u>	David E. Johnson	<u>41,874</u>
William Belanger	<u>40,509</u>	Christina Karnakis	<u>45,899</u>
Charles E. Bell	<u>48,128</u>	Kristin E. Konzak	<u>44,848</u>
Naomi Biswas	<u>38,384</u>	Cynthia Kozakiewicz	<u>42,764</u>
Bradford C. Blaise	<u>47,429</u>	Barry J. Marenberg	<u>40,715</u>
Sean M. Coughlin	<u>48,593</u>	Scott D. Miller	<u>43,803</u>
David F. Crosby	<u>36,400</u>	A. Jason Mirabito	<u>28,161</u>
Christopher J. Cuneo	<u>42,450</u>	Michel Morency	<u>50,183</u>
Brett N. Dorny	<u>35,860</u>	Carol H. Peters	<u>45,010</u>
Marianne Downing	<u>42,870</u>	Matthew Pavao	<u>50,572</u>
Ivor R. Elrifi	<u>39,529</u>	David Poirier	<u>43,007</u>
Heidi A. Erlacher	<u>45,409</u>	Michael Renaud	<u>44,299</u>
John M. Garvey	<u>37,833</u>	Brian Rosenbloom	<u>41,276</u>
James G. Gatto	<u>32,694</u>	Robert J. Sayre	<u>42,124</u>
Richard Gervase	<u>46,725</u>	C. Eric Schulman	<u>43,350</u>
Matthew J. Golden	<u>35,161</u>	Gregory J. Sieczkiewicz	<u>48,223</u>
Sonia K. Guterman	<u>44,729</u>	Thomas M. Sullivan	<u>39,392</u>
John A. Harre	<u>37,345</u>	Janine Susan	<u>46,119</u>
Brian P. Hopkins	<u>42,669</u>	Nicholas P. Triano III	<u>36,397</u>
		Howard Susser	<u>33,556</u>

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all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

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PATENT APPLICATION
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all of MINTZ, LEVIN, COHN, FERRIS, GLOVSKY AND POPEO PC, One Financial Center, Boston, Massachusetts 02111, as Applicant's attorneys with full power of substitution and revocation to take any and all action necessary with regard to the above-identified patent.

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